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Multiple origins of the common chameleon in southern Italy

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Abstract

The common chameleon *Chamaeleo chamaeleon* is a Mediterranean lizard which has been introduced in many islands and its native origin in European countries is debated. Chameleons have been introduced in southern Italy, possibly from the Middle East and Tunisia. We conducted genetic analyses on mitochondrial DNA 16S gene on a larger sample. We observed a multiple origin for the Salento (Apulia, southern Italy) population, with individuals phylogenetically related to populations of North Africa and two areas in the Middle East. Some individuals may have been released before the 1950s and some others in the 1980s, improving the establishment success of this species.

Key Words

16S rRNA gene, *Chamaeleo chamaeleon*, Mediterranean countries, paleo-introduction, population origin

Introduction

Genetics can play a crucial role in describing biological invasions, which are one of the major threats to global biodiversity (Vitousek et al. 1996; Schmitz and Simberloff 1997; Courchamp et al. 2017; Crooks et al. 2017). Accordingly, genetic variation can reveal taxonomic differences (Panzeri et al. 2014; Comtet et al. 2015; Mori et al. 2017; for a review, see Lawson Handley et al. 2011). A high genetic variability of the founders often results in a high colonization/invasion success (Mori et al. 2014; Jackson et al. 2015; Paulauskas et al. 2016). Genetics has been widely used to infer source populations and invasion paths (Stevenson-Holt and Sinclair 2015; Daane et al. 2018), which may help researchers to predict alien species' spread and inform authorities how to prevent further invasions (Ciosi et al. 2010; Mazzamuto et al. 2016).

Chameleons (Squamata, Chamaeleonidae) include over 210 lizard species grouped in 12 genera (Tilbury 2018). Among those, the genus *Chamaeleo* is the only one naturally present in Mediterranean countries, North Africa and the Middle East (www.iucnredlist.org. Downloaded on 29 April 2018). Traditional taxonomy identified four subspecies of the common chameleon *Chamaeleo chamaeleon* (Yaacov et al. 2012; Andreone et al. 2016; Tilbury 2018), which live in allopatry in its natural range. As to Europe, populations in the Iberian Peninsula seem to have been introduced from Maghreb, with multiple introduction events

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(Paulo et al. 2002). Common chameleons currently present in Cyprus and in Greek islands (e.g. Samos, Chios and Crete) share mitochondrial haplotypes with the Turkish population (Macey et al. 2008; Andreone et al. 2016). At least two subspecies of the common chameleon have been introduced to Malta (Dimaki et al. 2008; Andreone et al. 2016), one from Maghreb and the other from the Near East. As for southern Italy, chameleons in Calabria (province of Reggio Calabria) were possibly introduced from Tunisia (Andreone et al. 2016). Conversely, Andreone et al. (2016) showed that the population of the common chameleon in Salento (Apulia) may originate from the Middle East, sharing mitochondrial sequences with Eastern Mediterranean countries (northern Israel). In southern Italy, long-lasting observational data and records of young individuals provide strong evidence of reproduction and naturalization (Marzano and Scarafino 2010; Pellegrino et al. 2016). Other observations from the rest of Italy (Sicily, Sardinia, Latium, Liguria, Emilia Romagna and Friuli Venezia-Giulia) refer to single individuals (Bruno 1986; Andreone et al. 2016) and, for most of them, no photos or tissue samples or museum specimens exist (Andreone et al. 2016).

Andreone et al. (2016) analyzed three individuals from Salento, collected in the same year (2015), from the same study site (i.e., Nardò). According to published works, the first record of the common chameleon in Salento in the wild dates back to 1987 and, since then, records have increased (Basso and Calasso 1991; Marzano and Scarafino 2010). Andreone et al. (2016) and Marzano and Scarafino (2010) confirmed that most records came from the municipality of Nardò. Given that both Costa (1871) and Bruno (1986) did not report the presence of the chameleon in Salento, Andreone et al. (2016) claimed that the presence of the chameleon in this area might be the result of a recent introduction (e.g. early 1980s: see Scalera 2001). Conversely, Basso and Calasso (1991) already reported that the distribution of this small lizard could be even wider and possibly date back prior to 1980. In this work, we aimed at (*i*) mapping the local distribution of the common chameleon in Salento which underwent a range expansion, (*ii*) to identify the subspecies and (*iii*) to infer the origin of common chameleon established in this area.

Materials and methods

Redefinition of the distribution of the common chameleon in Salento

We collected all the available information on the presence of the common chameleon through field investigations, involving local rangers and human populations, as well as searching for grey literature, photos and other unequivocal presence signs of the common chameleon (e.g. individuals illegally collected by agricultural pruners of olive-trees) in Salento.

Molecular analyses

We extracted the DNA from tissue samples (i.e. tongue) of 18 museum specimens of the common chameleon collected by one of the authors (RB) between 1951 and 2017 (Appendix 1) from throughout the Salento peninsula, and stored in 95% ethanol at the Museo Civico di Storia Naturale di Jesolo (province of Venice, NE Italy). Individuals stored at the museum were found dead on paved road, road killed or preyed upon by domestic cats. Moreover, three of these samples belong to chameleons most likely poisoned by pesticides in olive groves (RB, unpublished).

We used the PureLink DNA Mini Kit (Invitrogen, by Thermo Fisher Scientific) and we followed the manufacturer's instructions. The reliability of DNA extraction was controlled through a negative control (no tissue added) and the DNA content determined through an Eppendorf Ultraviolet Spectrophotometer (AG Eppendorf). Mitochondrial DNA (mtDNA) genes, and particularly the 16 Svedberg rRNA gene (hereafter, 16S), show significant divergence among the four taxonomical subspecies of the common chameleon (Yaacov et al. 2012; Andreone et al. 2016), thus allowing researchers to infer the geographical origin of populations of this species.

We used species-specific primers to sequence a fragment of the mtDNA 16S gene, specifically designed on a consensus sequence using all the 16S sequences already present on GenBank, to increase the amplification success of our samples. We used the primers CAM-16S1 (5'-CCT GCC CTG TGG AAC CCT A-3') and CAM-16S2 (5'-GTT AGT CGT TGA ACA AAC GAA CCG-3'), specifically designed for this work. Retrieved sequences were aligned using MUSCLE (Edgar 2004). Conserved regions at both ends were used to design custom primers (Bio-Fab Research – Rome, Italy) with high melting temperature (above 60 °C) following Nerva et al. (2016). PCR reactions were run through a 2720 Thermal Cycler (Applied Biosystems), following this profile: 3 min 94 °C, 30 cycles of 35 s at 94 °C, 45 s at 62 °C and 3 min at 72 °C, followed by 7 min at 72 °C. PCR reactions were prepared following Mori et al. (2018), and using about 35 ng/ml of each DNA template. The electrophoresis was run for 45 min. on a 1.5% agarose gel.

Pairwise alignments of 16S sequences were performed using ClustalW (Higgins and Sharp 1988). The best fitting evolutionary model for further phylogenetic analysis was selected using the algorithm implemented in MEGA7 (Kumar et al. 2016). Tamura-Nei model with Invariant sites (TN93+I) resulted in the best model for the considered data. Evolutionary relationships were inferred with MEGA7 (Kumar et al. 2016), using the Neighbor-Joining and the Maximum Likelihood (1000 bootstrap replicates) methods based on the Tamura-Nei model (Tamura and Nei 1993).

We retrieved another 34 sequences of 16S rRNA gene of the common chameleon from GenBank (Table S2 in Appendix 1), which were used to compare ours. Subspecies distribution was drawn by using our sequences (N = 18) and those reported in the phylogenetic tree by Andre-

one et al. (2016) (N = 34 from throughout the known extent of occurrence of this species). The veiled chameleon *Chamaeleon calyptratus* was used as the outgroup.

Results

Distribution of the common chameleon in Salento (Apulia)

Local interviews and field-searches revealed that the current distribution of the common chameleon in Salento (Apulia) ranges between the municipalities of Nardò and Castrignano del Capo (Fig. 1), improving previous knowledge on its range. We found no evidence of the presence of the chameleon in Salento during the XVII–XIX centuries (Costa 1871). The earliest recent observations of common chameleons in Salento date back to the 1940s, in the municipalities of Calimera, Martano and Maglie, according to local coalminers interviewed by one of the authors (RB). Other observations were made south to Lecce, in coastline areas covered with Mediterranean scrubland, between 1940 and 1970. In 1951, a dead individual was collected and stored in a glass jar with ethanol and exposed in a chemist's shop in Lecce (Appendix 2). Since the 1980s, records of the common chameleon have increased in the number of records and sighting localities (Basso and Calasso 1991; Fattizzo and Marzano 2002; Marzano and Scarafino 2010; Andreone et al. 2016).

Figure 1. Distribution of the common chameleon in Salento (southern Italy) in 2018. Black dots represent observation localities.

Molecular analyses

Species-specific primers successfully amplified a ~380 bp DNA fragment. Chameleon samples from Salento clustered within the *C. c. chamaeleon* subspecies (N = 5 individuals), the *C. c. recticrista* subspecies (N = 2 indi-

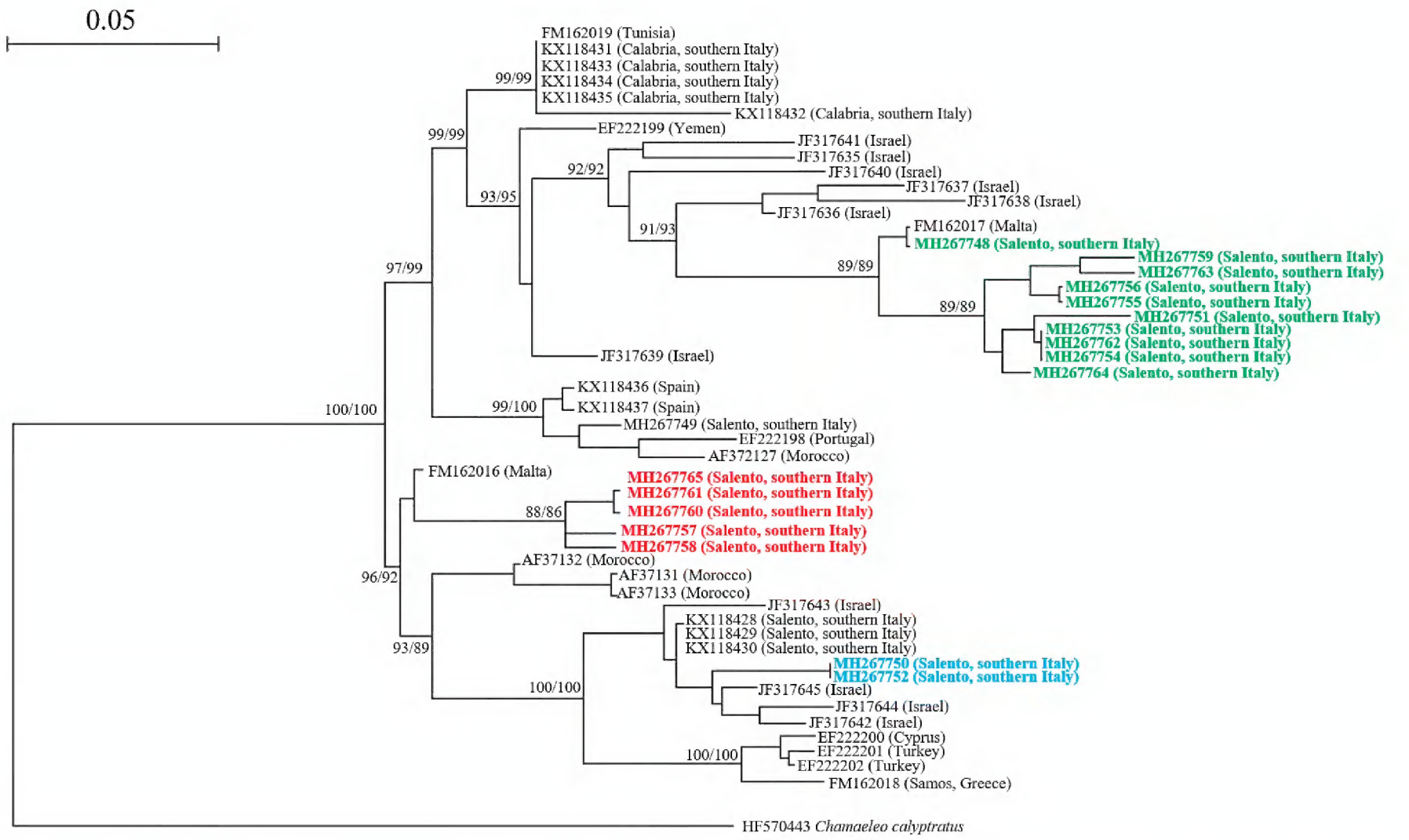

Figure 2. Neighbour-Joining phylogenetic tree of mtDNA sequences of the common chameleon. Numbers at nodes indicate, Neighbour-Joining and Maximum-Likelihood bootstrap values, respectively. Colored text showing samples included in this study: red, *C. c. chamaeleon*; orange, *C. c. recticrista*; green, *C. c. musae*.

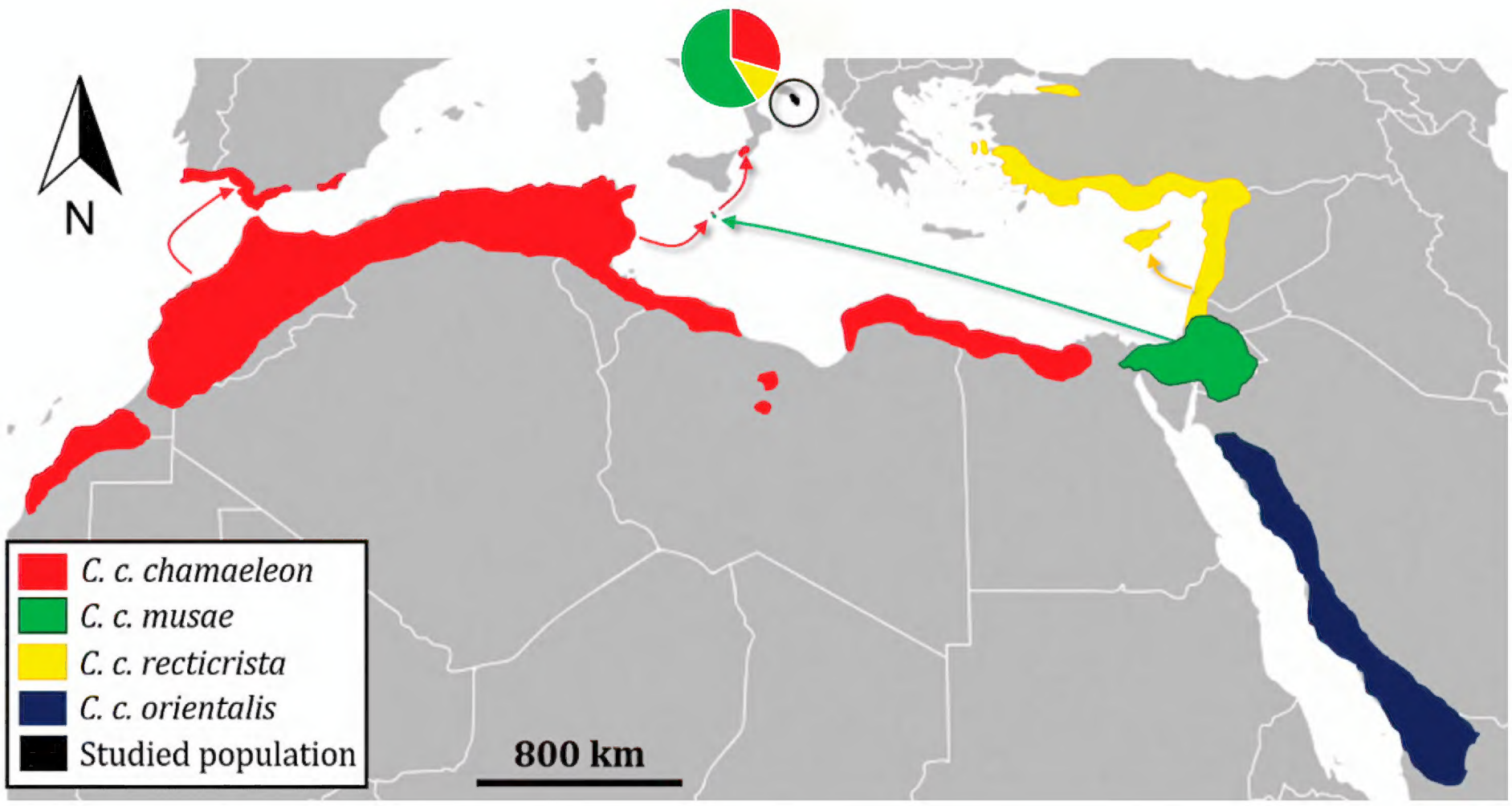

Figure 3. Distribution of the four subspecies of the common chameleon. Arrows show potential introduction pathways. The pie chart on Salento (southern Italy) summarizes the percentage of individuals of each subspecies detected in this area with our molecular analyses.

viduals) and, mostly, within the *C. c. musae* subspecies (N = 10 individuals), showing a multiple origin for this species in Salento (Figs 2 and 3). A total of 13 mtDNA 16S haplotypes were detected throughout the range of the common chameleon, three of them recorded in Salento (Fig. 3). Different subspecies were recorded even within the same localities, suggesting that they are intermixed in our study area, with no geographical local segregation (Table S1 in Appendix 1).

Discussion

Despite the high number of single records of this species throughout Italy in the wild (Andreone et al. 2016), only two established populations of the common chameleon seem to currently occur in southern regions, i.e. in Calabria and in Apulia (Basso and Calasso 1991; Pellegrino et al. 2016). The first is located in the province of Reggio Calabria, in the surroundings of Palmi (Pellegrino et al. 2016), whereas the second one has been only reported to occur in the surroundings of Nardò (province of Lecce, Salento, Apulia: Scalera 2001; Andreone et al. 2016). Our work showed that the distribution of the common chameleon in Salento is wider than previously known (i.e. potentially only the municipality of Nardò: Scalera 2001; Andreone et al. 2016), encompassing a 515 km^2 area on the Ionian coast between the municipalities of Nardò and Castrignano del Capo. The species is well-established and possibly expanding in this area, as confirmed by reports of reproductive females, eggs and juvenile individuals. Our genetic analyses also showed that chameleons currently present in Salento may belong to at least three subspecies. Andreone et al. (2016) reported that this lizard was introduced from Lebanon at the end of the 1980s; in our work, we showed that multiple introductions likely took place even before the 1980s, leading to the current lineage mix. According to our results, the common chameleon may represent an historical introduction in Salento, as already reported for the Iberian Peninsula (southern Spain and Portugal: Paulo et al. 2002) and Malta (Dimaki et al. 2008). Andreone et al. (2016) amplified very recent samples of common chameleon from Salento, which fell within the "*recticrista*" clade, together with Turkish, Samian, Cypriot and northern Israeli sequences.

The species originated in the Arab peninsula, with the subspecies *C. c. orientalis* being the oldest one, and evolved in Maghreb and southern Middle East; the most recent subspecies seems to be *C. c. recticrista* (Andreone et al. 2016). As to our samples, all the oldest samples belonged to Middle-Eastern mitochondrial clades (namely, "*recticrista*" and "*musae*": Yaacov et al. 2012), whereas a high number of the most recent ones belonged to the North African ("*chameleon*") clade. This confirmed our hypothesis of multiple releases in the study area.

Worldwide, apart from the common chameleon, alien chameleon populations are only represented by escapes of pet-chameleons (i.e. the veiled chameleon and the Oustalet's chameleon *Furcifer oustaleti* in Florida and the Jackson's chameleon *Trioceros jacksonii* in Hawaii: Krysko et al. 2004; Holland et al. 2010; Kraus et al. 2012; Smith et al. 2016). The veiled chameleon has been released to Florida since 2000 with a high propagule pressure (i.e. over 100 founders), but, despite its range is

growing (Gillette and Krysko 2012), ecological impacts on native environments have not been studied yet (Krysko et al. 2004). The introduction of the Jackson's chameleon to Hawaii dates back to the 1970s, where it might have affected native endangered invertebrates (e.g. *Achatinella mustelina*: Chiaverano and Holland 2014) and, in turn, breeding warblers and honeycreepers (Scalera 2001; Holland et al. 2010; Kraus et al. 2012). The establishment of the Oustalet's chameleon in the Everglades (South Florida) has been only recently assessed (Gillette et al. 2010; Smith et al. 2016) and data on the impact on native environments are not available. In the Hawaii archipelago, the veiled chameleon was eradicated in 2008, and a control program on the Jackson's chameleon is currently ongoing (Van Kleeck and Holland 2017).

Management of alien species, including alien chameleons, is pivotal for conservation, and eradication or numerical control are strictly required to prevent environmental alterations (Menchetti and Mori 2014; Pergl et al. 2016; Crowley et al. 2019). Alien species taxonomy and local ecology are paramount to build effective and reliable models to predict their range expansion and impacts (Sitzia et al. 2016; Buchadas et al. 2017; Davis et al. 2019; Mori et al. 2019). The ecology of the common chameleon in European countries, including its potential impacts on native species, is still not well known and, thus, it deserves further research in order to design addressed management plans.

Conclusion

Climatic matches and repeated introduction events may have favored the establishment success of the common chameleon in southernmost parts of Spain, Portugal, Greece and Italy, whereas introductions failed elsewhere (central Italy, northern Spain: Scalera 2001).

In Europe, although representing a historically introduced species, the common chameleon is strictly protected as it has long been considered a native taxon in all Mediterranean countries (Paulo et al. 2002). This species is listed within the Appendix II of the Bern Convention and within the Annex IV of the Habitat Directive (92/43/EEC), requiring a strict protection regime throughout its European extent of occurrence, in areas where it is believed to be native, e.g. Iberian peninsula, but not Italy, where it is claimed to be alien (Genovesi et al. 2014). Our results confirmed that introductions of this species in Italy have occurred before the 1980s. After pertinent ecological studies, management interventions may become necessary, in accordance with European guidelines (Genovesi et al. 2014; Pergl et al. 2016; Shackleton et al. 2019).

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RB, MS and EM conceived the idea. RB and MS collected the samples. MLV and EM carried out the genetic analyses and LN did the phylogenetic reconstructions. EM and GM wrote most of the paper. All authors participated in writing the final manuscript.

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Appendix 1

Table S1. Samples of common chameleon analysed in our work. ID Museum number, location and year of each sample, as well as accession numbers on GenBank are reported.

Museum ID	Location	Year	Subspecies	Accession number
CAM1	Gallipoli	1951	*C. c. musae*	MH267748
CAM20	Calimera	1989	*C. c. musae*	MH267764
CAM5	Gallipoli	1989	*C. c. recticrista*	MH267750
CAM11	Nardò	2009	*C. c. musae*	MH267756
CAM18	Nardò	2009	*C. c. chamaeleon*	MH267761
CAM10	Galatone	2010	*C. c. musae*	MH267755
CAM14	Ugento	2010	*C. c. musae*	MH267759
CAM17	Galatina	2011	*C. c. musae*	MH267762
CAM12	Supersano	2011	*C. c. chamaeleon*	MH267757
CAM16	Galatina	2012	*C. c. chamaeleon*	MH267760
CAM4	Nardò	2012	*C. c. chamaeleon*	MH267749
CAM6	Aradeo	2015	*C. c. musae*	MH267751
CAM7	Casarano	2016	*C. c. recticrista*	MH267752

Museum ID	Location	Year	Subspecies	Accession number
CAM13	Nardò	2016	*C. c. chamaeleon*	MH267758
CAM8	Taurisano	2016	*C. c. musae*	MH267753
CAM9	Taurisano	2016	*C. c. musae*	MH267754
CAM19	Gallipoli	2017	*C. c. musae*	MH267763
CAM15	Nardò	2018	*C. c. musae*	MH267765

Table S2. GenBank sequences of common chameleon used as a comparison with those in Tab. S1.

Origin	Subspecies	Accession number
Spain	*C. c. chamaeleon*	KX11436
Spain	*C. c. chamaeleon*	KX11437
Portugal	*C. c. chamaeleon*	EF222198
Morocco	*C. c. chamaeleon*	AF327127
Morocco	*C. c. chamaeleon*	AF327131
Morocco	*C. c. chamaeleon*	AF327132
Morocco	*C. c. chamaeleon*	AF327133
Calabria (Italy)	*C. c. chamaeleon*	KX118431
Calabria (Italy)	*C. c. chamaeleon*	KX118432
Calabria (Italy)	*C. c. chamaeleon*	KX118433
Calabria (Italy)	*C. c. chamaeleon*	KX118434
Calabria (Italy)	*C. c. chamaeleon*	KX118435
Tunisia	*C. c. chamaeleon*	FM162019
Malta	*C. c. chamaeleon*	FM162016
Malta	*C. c. musae*	FM162017
Israel	*C. c. musae*	JF317641
Israel	*C. c. musae*	JF317640
Israel	*C. c. musae*	JF317635
Israel	*C. c. musae*	JF317636
Israel	*C. c. musae*	JF317637
Israel	*C. c. musae*	JF317638
Israel	*C. c. musae*	JF317639
Salento (Italy)	*C. c. recticrista*	KX118428
Salento (Italy)	*C. c. recticrista*	KX118429
Salento (Italy)	*C. c. recticrista*	KX118430
Israel	*C. c. recticrista*	JF317645
Israel	*C. c. recticrista*	JF317642
Israel	*C. c. recticrista*	JF317643
Israel	*C. c. recticrista*	JF317644
Cyprus	*C. c. recticrista*	EF222200
Turkey	*C. c. recticrista*	EF222201
Turkey	*C. c. recticrista*	EF222202
Samos (Greece)	*C. c. recticrista*	FM162018
Yemen	*C. c. orientalis*	EF222199

Appendix 2

Figure S1. Samples of common chameleon used in our work. A-C) old samples stored at the Natural History Museum of Jesolo; D) a free-living female in an olive yard in the surroundings of Gallipoli (photo Emiliano Mori).